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RAPID COMMUNICATION

Chimeric Langat/Dengue Viruses Protect Mice from Heterologous Challenge with the Highly Virulent Strains of Tick-Borne Encephalitis Virus

Alexander G. Pletnev,*[,1] Galina G. Karganova,† Tatiana I. Dzhivanyan,† Vasilii A. Lashkevich,† and Michael Bray‡

*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; †Chumakov's Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow Region 142782, Russia; and ‡Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702

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Langat virus (LGT), a tick-borne flavivirus, is naturally attenuated for humans but it is very virulent in SCID mice. In contrast, viable recombinant chimeras of LGT (preM and E genes) and dengue type 4 virus (all other sequences) recovered in mosquito cell culture were completely attenuated in SCID mice but still capable of providing protection against LGT. To develop the chimeras into vaccine candidates, we adapted them to replicate efficiently in simian *Vero* cells, a satisfactory substrate for human vaccines. The adapted chimeras remained completely attenuated for SCID mice and, significantly, provided protection in immunocompetent mice against tick-borne encephalitis virus, the most virulent of the tick-borne flaviviruses.

The tick-borne virus complex of the *Flaviviridae* family includes tick-borne encephalitis (TBE), Kyasanur forest disease, Langat (LGT), Louping ill, Negishi, Omsk hemorrhagic fever, and Powassan viruses (for a review, see (*1*)). This group of viruses is endemic throughout most of the northern hemisphere and except for Langat virus causes human disease of varying severity. Viruses of this flavivirus complex are maintained in nature by a cycle involving ticks and wild rodent hosts. Based on vector transmissibility, serological analysis, and sequence analysis of isolates from Europe and Asia, TBE virus (TBEV) has been subdivided into two closely related subtypes, a European subtype (also referred to as Central European encephalitis) and a Far Eastern subtype (formerly called Russian spring–summer encephalitis) (*1–3*). Disease caused by TBEV is more severe in the Far East than in European countries, with a case fatality rate of up to 30% as well as neurologic sequelae, which occur in 30–60% of survivors. Currently, a vaccine produced by formalin inactivation of TBEV is available, but multiple inoculations are required to achieve a satisfactory level of resistance and the breadth of its protective effect has been questioned. For these reasons, we were interested in developing an improved vaccine against TBEV and other members of the tick-borne flavivirus complex.

Recently, we developed a new approach to immunoprophylaxis, which involved the construction of recombinant chimeric flaviviruses that were devoid of detectable neuroinvasiveness (*4–6*). These chimeric viruses contained the genes for structural proteins preM and E of TBEV or Langat virus, which were substituted for the corresponding genes of dengue type 4 virus (DEN4). This substitution was achieved using full-length infectious DEN4 cDNA (*7*). The resulting TBEV/DEN4 or LGT/DEN4 chimera exhibited a reduction in neurovirulence for mice, as tested by intracerebral inoculation. However, more impressive was the effect of chimerization on neuroinvasiveness, a property that reflects the capacity of a virus to replicate at a peripheral site and then spread to the central nervous system, where it causes encephalitis. Chimerization of TBEV or LGT with DEN4 completely ablated detectable neuroinvasiveness when assayed by the most sensitive indicator system, the SCID mouse. For example, peripheral inoculation of 10^7 plaque-forming units (PFU) of either chimera failed to produce encephalitis in SCID mice (*6, 8*). Also, in a previous study, a TBEV/DEN4 chimera that lacked neuroinvasiveness in SCID mice induced a protective immune response in normal mice that conferred complete resistance to challenge by homotypic, highly virulent TBEV (*4*). More recently we observed that the preM and E proteins of LGT in the LGT/DEN4 chimera provided significant protection when previously immunized mice were challenged intraperitoneally (ip) with wild-type LGT strain TP21 (*6*).

The tick-borne flaviviruses share envelope glycopro-

[1] To whom correspondence should be addressed at Building 7, Room 236, NIAID, NIH, 7 Center Dr. MSC 0740, Bethesda, MD 20892. E-mail: apletnev@niaid.nih.gov.

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tein epitopes that provide cross-resistance among viruses of this antigenically related group (*1, 9, 10*). This conservation of protective neutralization epitopes on the envelope glycoprotein includes Langat virus, a virus that is highly, but not totally, attenuated for humans. More than two decades ago this virus was evaluated as a candidate vaccine in over 600,000 persons in the former Soviet Union (*11*). At first the virus appeared to be completely attenuated, but the clinical trial was terminated when it became apparent that central nervous system signs developed at a rate of one such adverse reaction per 18,570 vaccines. Notably, the experimental vaccine virus induced durable immunity to TBE disease in endemic areas. In support of this finding, other studies indicated that immunization of experimental animals and human volunteers with Langat TP21 or E5 induced a high level of serum neutralizing antibodies against virulent members of the tick-borne flavivirus complex such as TBEV, Kyasanur forest disease virus of India, and Powassan virus of the northern U.S.A. and Canada (*9, 11, 12*). These serological responses provided direct evidence for conservation of protective epitopes among tick-borne flaviviruses, including the naturally attenuated Langat virus.

Although Langat virus (strain TP21) and its more attenuated chick embryo cell culture passage derivative, strain E5, were significantly attenuated for mice and monkeys, these viruses exhibited some neurovirulence (after intracerebral inoculation) and neuroinvasiveness (after peripheral inoculation) in mice, albeit less than that exhibited by TBEV (*6, 13, 14*). However, this residual reduced level of virulence of both strain TP21 and strain E5 was completely ablated by constructing viable chimeras with DEN4, in which the preM and E genes of TP21 or E5 were substituted for the corresponding genes of DEN4 (*6*).

Ablation of the residual neuroinvasiveness of the LGT strains TP21 and E5 suggested that one of these chimeras might prove to be useful as a live attenuated virus vaccine for prevention of disease caused by tick-borne flaviviruses. The present study addresses issues of the immunogenicity of the chimeric LGT/DEN4 vaccine candidates in mice and the ability of the chimeras to induce resistance to challenge with highly virulent TBEV.

Characterization of Vero Cell-Passaged LGT/DEN4 Chimeras. Two viable chimeric viruses that contained preM and E genes of wild-type LGT strain TP21 or its more attenuated derivative, LGT strain E5, with remaining sequences derived from DEN4 were recovered after transfection of mosquito C6/36 cells with full-length RNA transcripts of the full-length cDNA chimeric genome; however, infectious virus could not be recovered following transfection of simian cells (*6*). It should be noted that the former cells are considered to be unsuitable for preparation of human vaccines. Initially, both TP21/DEN4 and E5/DEN4 chimeras recovered in mosquito cells were significantly reduced in efficiency of viral replication and plaque formation in simian cells compared with parental TP21 or E5 virus as well as parental DEN4. However, it was possible to adapt the chimeric viruses to grow efficiently in certified *Vero* cells (W.H.O. Seed, 143 passage; Novavax, Inc., Rockville, MD) suitable for use in production of human vaccines. This was accomplished by inoculating *Vero* cells with TP21/DEN4 or E5/DEN4 virus at a multiplicity of infection of 1 or 5 and harvesting 2- to 4.5-mm virus plaques that developed after 10 days of incubation at 37°C. These plaque isolates were then subjected to four plaque to plaque passages in *Vero* cells in a successful attempt to select for virus that grew to higher titer and produced plaques more efficiently. Seed stock of *Vero* cell culture-derived TP21/DEN4 or E5/DEN4 virus was prepared by passage of fourth-plaque-passage virus in *Vero* cells.

The *Vero* cell-adapted vaccine candidates (vac) TP21/DEN4(vac) and E5/DEN4(vac) were then compared with each other and with their parental viruses with respect to neuroinvasiveness in mice, plaque morphology, and maximum yield in simian and mosquito cells. The titers attained by the *Vero* cell-adapted TP21/DEN4(vac) and E5/DEN4(vac) chimeras were 4.6×10^6 and 3×10^6 PFU/ml in *Vero* cells and 1×10^6 and 3×10^6 PFU/ml in mosquito C6/36 cells, respectively, indicating that parity had been achieved.

The increased cytopathic effect of the *Vero* cell-adapted chimeras in *Vero* cells suggested that host range mutations in the virus genome were selected during adaptation and propagation of these viruses in simian cells. For this reason a partial sequence of each chimeric virus genome was determined by RT–PCR analysis of RNA extracted from purified virions to verify their chimeric structure and identify mutations that might play a role in *Vero* cell adaptation. Primer pairs (oligo 239 and oligo 442; see (*5*)) that amplify the DEN4 genome from nucleotide 18 to nucleotide 2832 were used to generate PCR products. The nucleotide sequence of the 5′ noncoding region, the structural protein genes, and the nonstructural protein NS1 gene of each *Vero* cell culture-derived chimeric genome, including the C/preM and E/NS1 junctions, was determined and compared with the published sequence of the corresponding mosquito cell culture-derived chimeric virus genome (Table 1) (*6*). Only three amino acid differences were identified and these were located in the E protein sequence of the TP21/DEN4(vac) chimera that had been passaged five times in *Vero* cells following recovery in mosquito C6/36 cells. In a similar comparison, there were only eight nucleotide differences in the sequence of the *Vero* cell-adapted E5/DEN4(vac) chimera, of which six produced an amino acid substitution, and these were located in the preM and envelope structural protein (E). The same amino acid substitutions in E at position 296 (Lys → Gln) and posi-

TABLE 1

Mutations Acquired When TP21/DEN4 and E5/DEN4 Chimeras Recovered in Mosquito Cells Were Adapted to Grow Efficiently in Simian *Vero* Cells

Region of viral genome	Nucleotide position	TP21/DEN4 virus grown in		E5/DEN4 virus grown in		Amino acid change
		C6/36 cells	*Vero* cells[a]	C6/36 cells	*Vero* cells[a]	
C	212[b]			U	**C**	
Pre-M	643			G	**A**	
	870			U	**C**	$Phe_{151} \rightarrow Ser$
E	1437			C	**U**	$Thr_{151} \rightarrow Ile$[c]
	1856	A	**C**	A	**C**	$Lys_{296} \rightarrow Gln$
	1898	A	**G**	A	**G**	$Thr_{310} \rightarrow Ala$
	1973			A	**U**	$Thr_{335} \rightarrow Ser$
	2371[b]	G	**U**			$Cys_{480} \rightarrow Phe$
	2403[b]			G	**A**	$Gly_{491} \rightarrow Ser$

[a] Virus recovered from cDNA in mosquito C6/36 cells and then passaged five times in simian *Vero* cells.
[b] Nucleotide number for DEN4 genome.
[c] Results in loss of potential glycosylation site.

tion 310 (Thr → Ala) were documented in the two chimeras. These shared mutations may play a role in alteration of cell tropism.

The change at nucleotide position 1437 of the E5/DEN4(vac) genome results in an amino acid substitution of Ile for Thr_{151} in a potential glycosylation site of the E protein. Immunoprecipitation of viral E proteins from lysates of *Vero* cells infected with either a parental LGT virus or its chimeric virus indicated a difference in gel migration of the E protein of parental E5 virus and its chimeric E5/DEN4(vac) virus (data not shown). The E protein of the E5/DEN4 chimera migrated slightly faster than the E protein of the E5 virus. This probably reflects loss of one of the three potential N-linked glycosylation sites in the E protein. In contrast, gel mobilities of the E glycoprotein of TP21 and its chimeric TP21/DEN4(vac) virus did not differ.

Mouse Neuroinvasiveness. In a previous study, LGT TP21 inoculated ip was only moderately attenuated for immunocompetent mice (*6*). In contrast, LGT E5 (an egg-passage derivative of TP21), TP21/DEN4, and E5/DEN4 were completely attenuated when normal mice were inoculated by the ip route. However, the parental LGT viruses inoculated ip exhibited a very high level of virulence for SCID mice; the ip LD_{50} was 4×10^{-3} PFU for TP21 and 6×10^{-2} PFU for E5 (Table 2) (*6*). Significantly, this high level of virulence of the LGT strains for SCID

TABLE 2

Neuroinvasiveness of *Vero* Cell-Grown LGT/DEN4 Chimeras Used for Immunization

Mice	*Vero* cell-grown LGT strains and LGT/DEN4 chimeras tested: Virus	Amount of virus inoculated ip as determined in *Vero* cells (PFU)	% mortality (No. dead/no. tested)	Estimated LD_{50} (PFU)	Previously determined neuroinvasiveness (LD_{50}) of mosquito cell-grown LGT/DEN4 chimeras[a]
Swiss	TP21/DEN4(vac)	5×10^5	0 (0/10)	$>5 \times 10^5$	$>10^5$
	E5/DEN4(vac)	5×10^5	0 (0/5)	$>5 \times 10^5$	$>10^5$
	TP21	1×10^5	100 (10/10)	$<10^5$	5×10^3
	E5	1×10^5	0 (0/5)	$>10^5$	$>10^7$
SCID	TP21/DEN4(vac)	5×10^5	0 (0/10)[b]	$>5 \times 10^5$	$>10^7$
	E5/DEN4(vac)	5×10^5	0 (0/10)[b]	$>5 \times 10^5$	$>10^7$
	TP21	1×10^2	100 (10/10)	$<10^2$	4×10^{-3}
	E5	1×10^2	100 (10/10)	$<10^2$	6×10^{-2}

[a] Ip LD_{50} (PFU) determination based on titration in mosquito cells (Ref. 6). LGT strains TP21 and E5 were titered in *Vero* cells. Data from Ref. 6 presented for purpose of comparison.
[b] Five surviving mice were challenged ip on day 50 with 10^2 PFU of TP21 virus. All mice died 10 to 13 days postchallenge. Brain, liver, and blood of the five remaining survivors were collected on day 50 and tested for viral genome by direct RT–PCR analysis and for viable virus by inoculation of *Vero* cell cultures that were analyzed by immunofluorescence for viral antigens 7 days later.

mice was totally ablated when TP21 or E5 was used to construct a viable LGT/DEN4 chimera. In a previous study, the ip LD_{50} in SCID mice for the two chimeras "rescued" and propagated in mosquito cells was $>10^7$ PFU (*6*). In the present study a similar analysis was performed for the *Vero* cell-adapted chimeras to determine if this level of attenuation for SCID mice had been retained after the chimeras were adapted to certified *Vero* cells that are qualified for use in the manufacture of a human vaccine.

Three-week-old Swiss mice and immunodeficient (SCID) mice (C.B.-17 ICR/scid/scid; Taconic Farms, Germantown, NY) were used to assess virulence (Table 2). At a dose of 10^5 PFU delivered ip, parental TP21 caused 100% mortality in Swiss mice, whereas LGT E5, the TP21/DEN4(vac) chimera, and the E5/DEN4(vac) chimera failed to cause fatal disease when inoculated ip at a dose of 10^5 or 5×10^5 PFU. In addition, complete attenuation of both chimeric viruses was observed when SCID mice were inoculated ip. Although TP21 and E5 viruses produced 100% mortality when SCID mice were inoculated ip with 10^2 PFU, neither mortality nor illness was observed when SCID mice were inoculated ip with 5×10^5 PFU of either LGT/DEN4 chimera. This indicates that *Vero* cell-adapted viruses, like their mosquito cell-grown parents, were significantly attenuated for mice under these conditions.

To determine if SCID mice that survived 7 weeks after ip inoculation with either LGT/DEN4 chimera were susceptible to challenge with parental TP21, surviving mice were inoculated ip with 10^2 PFU of TP21. As expected, all of these mice died between 10 and 13 days postinoculation. We were also unable to recover infectious virus or detect viral RNA by PCR 50 days after ip inoculation of either highly attenuated chimera. Thus, we were unable to detect evidence of persistent infection.

Protection against Challenge with Virulent TBEV. The studies involving TBEV challenge were carried out in a biosafety level 4 laboratory at the Chumakov's Institute of Poliomyelitis and Viral Encephalitides, Moscow region, Russia, or at the U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD) in accordance with procedures described in the *Guide for the Care and Use of Laboratory Animals* (*18*).

Protection Induced by Immunization with a 600 PFU Dose of the TP21/DEN4 Chimera (Table 3). In the first experiment, 6-week-old inbred CBA mice (14–20 g) in groups of 9 or 10 were inoculated ip with 600 PFU of TP21/DEN4(vac) once or twice with an interval of 29 days between inoculations. Immunized as well as nonimmunized (control) mice were challenged ip on day 26 or 55 with 320 PFU ($32LD_{50}$) of the highly neuroinvasive TBEV strain Absettarov, a European subtype virus (*3*). The ip LD_{50} of this strain for 14- to 20-g CBA mice was estimated to be 10 PFU. Mice immunized with a single 600 PFU dose of the chimeric vaccine were only partially protected (60%) against $32LD_{50}$ of the virulent TBEV strain, whereas two inoculations of a 600 PFU dose of the chimera conferred complete protection against heterologous challenge with TBEV. In contrast, all 19 control CBA mice developed clinical signs consistent with lethal TBEV infection and died when challenged with TBEV.

During the second experiment, a similar protocol was used to study the TP21/DEN4 chimera in BALB/c mice. Four-week-old BALB/c female mice (10–14 g) in groups of 5 or 10 were inoculated ip with 600 PFU of TP21/DEN4(vac) chimera one or more times with an interval of 26 to 66 days between inoculations (Table 3). Mice were challenged ip at the indicated time(s) with a dose of $320LD_{50}$ of TBEV strain Absettarov, for which the ip LD_{50} for 10- to 14-g BALB/c mice was estimated to be 1 PFU. In this experiment as well as the first experiment, nonimmunized mice that served as controls were the same age as immunized mice to eliminate an effect of age-related resistance of mice to TBEV. All 40 nonimmunized mice challenged with TBEV died with clinical signs of lethal TBEV infection. BALB/c mice inoculated with a single dose of the TP21/DEN4(vac) chimera were poorly protected against TBEV compared to CBA mice. However, protective efficacy increased when two or three doses of the chimeric vaccine strain were given. Complete protection to TBEV challenge was achieved when the vaccine candidate was inoculated four times over a period of 127 days.

Protection Induced by Inoculation with a 10^5 PFU Dose of a Candidate LGT/DEN4 Vaccine (Table 4). Three-week-old outbred Swiss female mice (7–9 g) were inoculated by the ip route with (i) 10^2 PFU of LGT TP21 virus, LGT E5 virus, or a cDNA-derived LGT TP21 virus (designated 656) (*8*) or (ii) 10^5 PFU of TP21/DEN4(vac), E5/DEN4(vac), or DEN4. When the candidate vaccine was administered twice, the second inoculation was given after an interval of 22 days. Eighteen days after the second immunization, all of the mice were bled to measure the level of serum neutralizing antibodies against LGT TP21 virus, and 6 days later mice were challenged ip with a $100LD_{50}$ of the highly virulent strain Sofjin of TBEV, Far Eastern subtype (*2*). The 50% lethal dose of the Sofjin strain for 8-week-old mice was previously determined to be 0.5 PFU (*10*).

Previous studies in mice demonstrated a tight correlation between the level of serum neutralizing antibodies to tick-borne flavivirus induced by immunization and the resistance to challenge with homologous virus or other closely related members of this group (*1, 9, 10*). These earlier studies established that a close antigenic relationship existed between TBEV and LGT. Also, the sequences of the structural proteins preM and E of these flaviviruses are 85–88% homologous (*15*). Thus, we were not surprised to observe that the level of neutralizing

TABLE 3

Intraperitoneal (ip) Immunization of Inbred Mice with Low Dose of Langat TP21/DEN4(vac) Chimera Protects against Subsequent ip Challenge with Highly Virulent TBEV Strain Absettarov

ip immunization	No. of inoculation(s) (interval in days between inoculations)	Mouse strain	ip challenge with 320 PFU of TBEV on indicated day: Day of challenge	No. of mice	No. that survived (%)	Average survival time of mice that died (days)
600 PFU of TP21/DEN4(vac) chimera	1	BALB/c	26	10	2 (20)	9.8
		CBA	26	10	6 (60)	11.2
	2 (29)	BALB/c	55	10	4 (40)	9.7
		CBA	55	9	9 (100)	
	2 (26)	BALB/c	94	10	6 (60)	12.5
	3 (29, 39)	BALB/c	94	10	8 (80)	16
	4 (26, 66, 35)	BALB/c	180	5	5 (100)	
Tissue culture medium		BALB/c	26	10	0 (0)	8.1
			55	10	0	
			94	10	0	
			180	10	0	
		CBA	26	9	0	10.2
			55	10	0	

antibodies measured against LGT TP21 virus showed a correlation with protective immunity to TBEV. In this experiment the LGT virus-specific immune response of mice was measured by determining the titer of serum LGT TP21 neutralizing antibodies induced by the chimeric vaccine candidate or its parental LGT virus. Individual serum samples were analyzed by a 50% focus reduction neutralization test (*16, 17*) using TP21 virus (Table 4). Mice inoculated once with 10^2 PFU of TP21, TP21(656), or E5 virus or twice with 10^5 PFU of the TP21/DEN4(vac) chimera developed a high level of neutralizing antibodies against LGT TP21. In contrast, mice inoculated ip with 10^5 PFU of DEN4 failed to develop TP21 neutralizing antibodies. Also, mice immunized with either chimeric virus once or E5/DEN4(vac) chimera twice developed a moderate level of TP21 serum neutralizing antibodies, which was lower then observed previously (*6*), when the mosquito cell culture-derived chimeric viruses were used for immunization of outbred Swiss mice.

TABLE 4

Intraperitoneal (ip) Immunization of Swiss Mice with Langat (LGT)/DEN4 Chimeras Protects against Subsequent ip Challenge with Highly Virulent TBEV Strain Sofjin

ip immunization: Virus	Dose (PFU)	No. of inoculations (interval in days between inoculations)	Serum-neutralizing antibody titer[a] (reciprocal geometric mean)	ip challenge with 100 LD_{50} of TBEV on day 46: No. mice	No. that survived (%)	Average survival time of mice that died (days)
TP21	10^2	1	1676	5	5 (100)	
TP21/DEN4(vac)	10^5	1	54	9	2 (22)	10.7
		2 (22)	452	6	6 (100)	
E5	10^2	1	489	10	10 (100)	
E5/DEN4(vac)	10^5	1	56	8	1 (12)	12.9
		2 (22)	96	6	4 (67)	11.0
TP21-656[b]	10^2	1	831	10	9 (90)	10.0
DEN4	10^5	1	<20	8	0 (0)	10.6
Tissue culture medium	—		<20	19	0 (0)	10.7

[a] Neutralizing antibodies in mouse serum collected 40 days after first immunization were measured by a 50% focus reduction neutralization test using TP21 virus.

[b] Mutant of cDNA-derived LGT strain TP21 (*8*).

Mice previously inoculated with a low dose of E5 or TP21 were fully protected against subsequent TBEV challenge, whereas mice previously inoculated with DEN4 as well as nonimmunized mice were not protected at all. This indicates that protection was due to a broad LGT immune response. Even a very low dose of live LGT virus (including the cDNA-derived TP21 virus) was highly effective in preventing disease caused by the antigenically related TBEV. Mice also became resistant to subsequent lethal challenge with the Far Eastern subtype strain of TBEV after immunization with the chimeric viruses. The TP21/DEN4(vac) appeared to be more immunogenic than E5/DEN4(vac) because mice inoculated with two doses of the former were fully protected against TBEV challenge. In contrast, only 67% of mice inoculated with two doses of E5/DEN4(vac) chimera survived lethal challenge by TBEV. Clearly, the parental LGT viruses were more immunogenic and protective than their DEN4 chimeras. However, it was possible to achieve greater safety and equivalent protective efficacy when the TP21/DEN4(vac) chimera was administered in a two-dose regime.

Consistent with the close antigenic relationship of LGT and TBEV, our studies with chimeric virus vaccine candidates in mice have shown a high degree of cross-protection between LGT and the TBEV European subtype (strain Absettarov) or the TBEV Far Eastern subtype (strain Sofjin). Thus, the LGT preM and E proteins of the chimeras represent effective protective antigens able to induce significant resistance to heterologous challenge with highly virulent TBEV. The encouraging results obtained from analysis of the safety, immunogenicity, and protective efficacy of the LGT/DEN4 chimeric candidate vaccine strains in mice constitute a basis for expanding our evaluation of the vaccine candidate to nonhuman primates. As a consequence, dose escalation and immunogenicity studies are underway in monkeys.

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